GENE EXPRESSION PROFILE AND COMPARATIVE STUDY OF MBD GROUP OF PROTEINS IN LEUKEMIA SAMPLE

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Submitted by

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CERTIFICATE

This is to certify that the thesis entitled **"Gene Expression Profile & Comparative Study of MBD Group Proteins in Leukemia Sample"** which is being submitted by Miss Saswati Swain, Roll No. 410LS2078, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

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DECLARATION

I, Saswati Swain, hereby declare that this project report entitled **"Gene Expression Profile & Comparative Study of MBD Group of Proteins in Leukemia Sample"** is the original work carried out by me under the supervision of Dr. Samir K. Patra, Associate Professor and Head, Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge and belief the present work or any other part thereof has not been presented to any other University or Institution for the award of any other degree.

Date: **Saswati Swain**

Place:

ACKNOWLEDGEMENT

Accomplishment is a word which always sounds good. It is really a pleasure to see things done in a perfect and orderly manner. I take this opportunity to offer my gratitude to every person who has been a part of this project.

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I am genuinely appreciative of all my batch mates for their suggestions and moral support during my work.

Last but not the least, I would not have been able to complete this project without the love and support of my parents whose unflinching faith in my abilities helped me to overcome many obstacles and march ahead in spite of failures. I would like to dedicate this project to my parents and my sister.

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ABBREVIATIONS

µ	Micro
:	Ratio
%	Percentage
µl	Micro liter
µg	Microgram
Bp	Base pare
cDNA	Complimentary DNA
DNA	Deoxy Ribonucleic Acid
dNTP	Deoxy Nucleotide Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
gm	Gram
Fig.	Figure
MBD	Methyl-CpG-binding domain;
MeCP2	Methyl-CpG-binding protein 2;
TRD	Transcriptional repression domain;
CXXC	Cysteine rich domain;
GR	Glycine and arginine

Viz.	Such as
PCR	Polymerase Chain Reaction
dATP	2’-deoxyadenosine 5’-triphosphate
dCTP	2’-deoxycitidine 5’-triphosphate
dGTP	2’-deoxyguanosine 5’-triphosphate
DEPC	Diethyl Pyrocarbonate
GC	Guanine and Cytosine
OD	Optical density
UV	Ultra Violet
mRNA	Messenger RNA
RNase	Ribonuclease
RT	Reverse Transcriptase
RPM	Revolution Per Minute
TBE	Tris Borate EDTA
U	Unit
et.al	And others

CONTENTS

ABSTRACT

DNA methylation is the principle epigenetic modification mediating cell-specific functional variation in the phenotypic expression of the genome. This phenomenon is essential for genome maintenance and chromosomal integrity. The main players in this scheme are the DNA methyltransferases DNMT1, DNMT3A, DNMT3B who faithfully replicate the pre-existing methyl marks and propagate the methylation patterns across successive cell generations. The DNMTs are supported in their function by the methyl binding domain proteins called MBD proteins. This family of proteins preferentially binds methylated CpG dinucleotides, hinder the interaction of transcription factors with the DNA and prevent assembling of the transcriptional mechanism, thus result in heterochromatinization and transcriptional silencing. The functional significance of the MBD proteins in the transcriptional regulatory system necessitates the need to understand the involvement of these proteins in the epigenetic context. Keeping this observation in mind, the present study was designed to investigate the expression pattern and gene profiling of MBD proteins (MBD1, MBD2, MBD3 and MBD4) so as to conduct a comparative analysis of their relative levels of expression in leukemia samples. The project aims to provide a comprehensive knowledge about the expression of the MBD proteins in cancer samples in order to create a distinct epigenetic biomarker for effective prognosis of cancer. Further studies in a variety of different cancer tissues will provide an exhaustive idea regarding the role of MBD proteins in malignant transformation and subsequently result in an epigenetic signature for efficient molecular detection strategies.

Keywords: Epigenetics, DNA methylation, MBD proteins, Cancer, Leukemia.

INTRODUCTION

INTRODUCTION

Cancer is a process driven by the growth of abnormality in gene function; although many of these changes are genetic, epigenetic changes in gene expression are being progressively more appreciated. This process involves two components of heritable, but reversible, accent of gene promoter utility that are closely tied to one another – formation of chromatin which modulates transcription and establishing patterns of DNA methylation. Epigenetic modifications that affect gene expression include post-translational modifications on N-terminal tails of histones and DNA methylation. Detailed investigation of epigenetic status can provide insight into the position and function of genetic regulatory elements on a genome-wide origin .A major dispute in the post-genomic era is the mapping and functional characterization of the full balance of transcriptional regulatory elements as well as promoters and enhancers also locus control regions (LCRs) and chromatin domain frontier elements. Epigenetics, the cell-type-specific interpretation of genetic material, present on DNA methylation and post-translational histone modifications to control gene function.

DNA (cytosine-5) methylation is known to be an evolutionary process and affects lively chromatin formation (Patra, et al., 2001). The enzyme responsible for DNA methylation is DNA methyltransferase (DNMT). Many isoforms (i.e., DNMT 1, 2, 3a, 3b, and 3L) of human DNMT be now recognized and known to perform a specific pattern of methylation [T.H. Bestor, 2000]. DNA methylation is a vital for suitable chromatin structure and function in mammalian cells. DNA methylation is a quintessential process in cellular context helping in parental imprinting, transposon silencing, chromosome dosage compensation, and also genome stability. Aberrant hypermethylation of promoter CpG islands and the resulting transcriptional silencing is nowadays a widely accepted mechanism of inactivation of tumor suppressor genes in cancer that actively contributes to tumorigenesis (Jones and Laird, 1999; Herman and Baylin, 2003*).* One of the key achievements in cancer epigenetics has been the recognition of profiles of aberrant CpG hypermethylation that are specific to the tumor type (Costello, et al., 2000; Esteller, et al., 2001*).* The survival of these profiles provide a powerful set of markers for outlining the disruption of critical pathways in tumorigenesis and for deriving sensitive molecular detection strategies for virtually every human tumor category. The orderly study of DNA methylation patterns in human

cancer cell lines has shown that these are appropriate models for this type of study as they show methylation patterns that resemble their corresponding tumor types *(*Smiraglia, et al., 2001; Paz, et al., 2003).

Classical methyl-CpG binding proteins contain the conserved DNA binding motif methyl cytosine binding domain (MBD),which preferentially bind to methylated CpG dinucleotides. These proteins provide as transcriptional repressors, mediating gene silencing by means of DNA cytosine methylation. Mutations within methyl-CpG binding protein 2(MeCP2) have been associated to the human mental retardation disorder Rett syndrome, suggesting an important function for methyl- CpG binding proteins in brain development and function.$MeCP_2$ recruits histone modifying proteins which aid in the formation of the heterochromatin (inactive chromatin). An additional influence of methylated DNA on gene transcription is due to the hindrance offered by the methylated cytosine residue which interferes with transcription factor binding and assembling of the transcriptional mechanism (Takizawa, et al., 2001).

DNA methylation is the major modification of eukaryotic genomes and plays an essential function in mammalian progression. Human proteins MECP2, MBD1, MBD2, MBD3 and MBD4 encompass a family of nuclear proteins related by the presence in each of a methyl-CpG binding domain (MBD). Each of these proteins, with the exemption of MBD3, is capable of binding specifically to methylated DNA. MECP2, MBD1 and MBD2 can in addition repress transcription from methylated gene promoters. In contrast to other MBD family members, MECP2 is X-linked and subject to X inactivation. MECP2 is not necessary in stem cells. MECP2 gene mutations are the reason of most cases of Rett syndrome, a rapid neurologic developmental disorder and one of the most common causes of mental retardation in females. In the light of the functional significance of the MBD proteins in the epigenomic landscape, the present study was designed to investigate the expression pattern and gene profiling of this family of proteins (MBD1, MBD2, MBD3 and MBD4) so as to study their relative levels of expression in leukemia samples. The project aims to provide a comprehensive knowledge about the gene expression of the MBD proteins in cancer samples so as to create a distinct epigenetic biomarker for effective prognosis. Further studies in a variety of different cancer tissues will provide an exhaustive idea regarding the role of MBD proteins in malignant transformation and subsequently result in a epigenetic signature for efficient molecular detection strategies.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Epigenetics [Epi-above/over/beyond; above genetics] is the study of heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the underlying DNA sequence that result in cell specific functional variations in phenotype. It involves functionally significant modifications to the genome that does not include a change in the nucleotide chain. Examples of such changes are DNA methylation and Histone deacetylation, both of which suppress gene expression. Epigenetic variations in gene expression do not result in changes in the nucleotide sequence of DNA and consequently are not mutations. The study epigenesis which refers to hypothetical aspects of developmental biology and the strategy of genes was given by C.H.Waddington during 1930-1960.epigenetic modifications are heritable, reversible covalent modifications that work in tandem to orchestrate the transcriptional activity of the genome in various biological settings. These modifications include DNA methylation, Histone modifications and Non-coding RNA mediated gene regulation. Of these three, DNA methylation is the first and most extensively researched epigenetic manipulation.

2.1. DNA Methylation:-

Cytosine methylation at the 5-carbon position is the only known constant base modification seen in the mammalian genome. The organization and modification of chromatin is a key feature in programming gene expression models. Recent discoveries suggest that DNA methylation at the junction of transcription beginning and elongation plays a critical role in suppression of transcription. This effect is mechanistically mediated by the state of chromatin modification. DNA methylation attracts binding of methyl- CpG-binding domain proteins that trigger repression of transcription, whereas DNA demethylation facilitates transcription activation. It is paradox that DNA is a very stable molecule whose structure is faithfully maintained from generation to generation, yet with each round of replication, this structure is modified by base methylation in nearly all cells and organisms. The consequences of this modification are obviously of vital concern for all biologists. As well as the four major bases, the DNA of most organisms contains one or more minor bases. The commonest of these is 5-methylcytosine, but N-methyl adenine and N4-methylcytosine are also found, particularly in

prokaryotes. In vertebrates 3-6% of DNA cytosine is methylated, but this importance decreases going down the evolutionary extent so that in many insects and single-celled eukaryotes there is no noticeable 5-methylcytosine. In contrast, plants may have 3000 of their DNA cytosine methylated (Adams & Burdon, 1985).

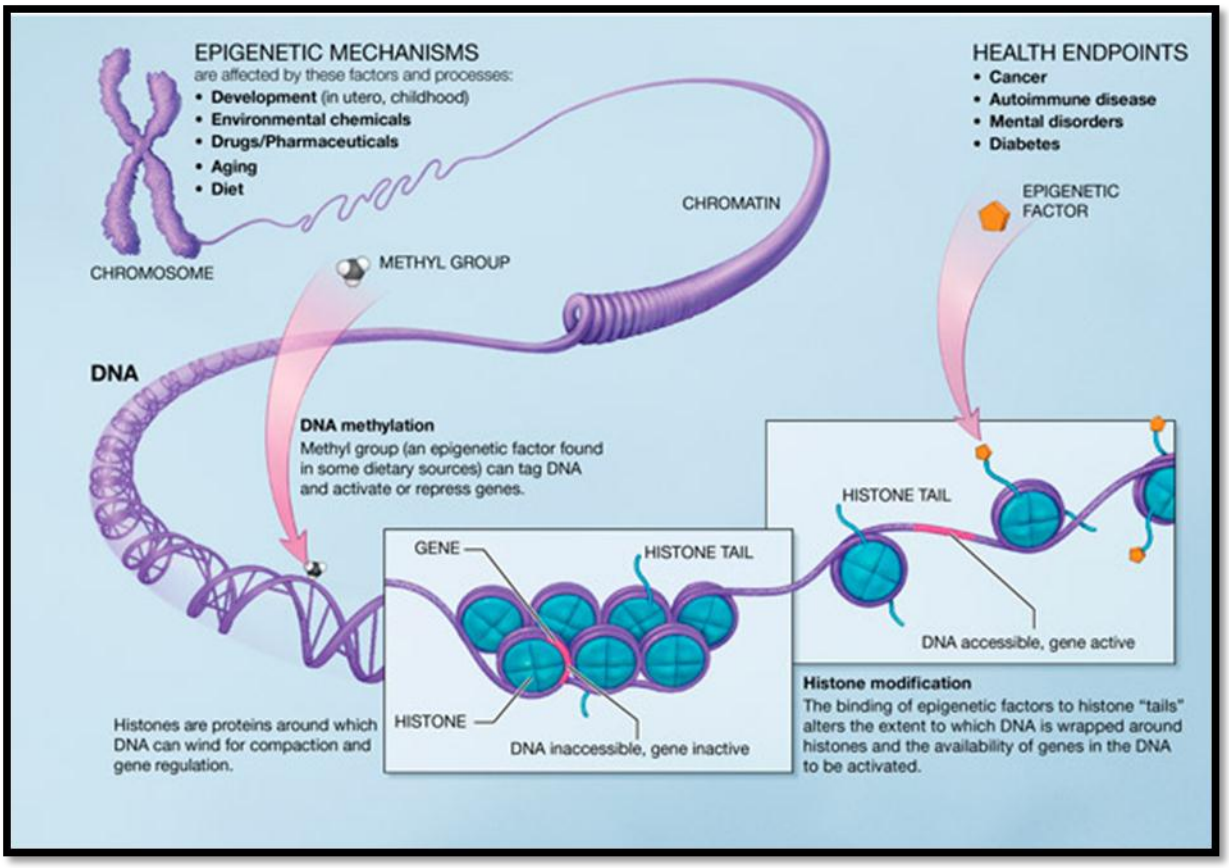


Fig 1: Epigenetic mechanisms are affected by several factors and processes (Adapted from Adams, R. L. P. & Burdon, R. H., Springer link, 1985).

DNA (cytosine-5-carbon) methylation is one of the hallmarks of mammalian chromatin modifications. Distinct methyl can generate synergistic or antagonistic interaction affinities before CpG-islands associated with methylated or unmethylated cytosine binding proteins, which may also dictate histone modification and dynamic transition between transcriptionally silent or transcriptionally active chromatin states (Patra et al,2008). In zygote development just after fertilization, parental DNA experiences drastic loss of DNA-methylation before the zygote starts

dividing (before its DNA is replicated).The enzyme and cofactors involved are still unknown. However the egg-derived DNA remains mostly methylated (Patra et al., 2008).

DNA methylation is the major modification of eukaryotic genomes and plays an essential role in mammalian development. Human proteins MECP2, MBD1, MBD2, MBD3, and MBD4 comprise a family of nuclear proteins related by the presence in each of a methyl-CpG binding domain (MBD). Each of these proteins, with the exception of MBD3, is capable of binding specifically to methylated DNA. MECP2, MBD1 and MBD2 can also repress transcription from methylated gene promoters. Five transcript variants of the MBD1 are generated by alternative splicing resulting in protein isoforms that contain one MBD domain, two to three cysteine-rich (CXXC) domains, and some differences in the COOH terminus. All five transcript variants repress transcription from methylated promoters; in addition, variants with three CXXC domains also repress unmethylated promoter activity. MBD1 and MBD2 map very close to each other on chromosome 18q21.

2.2. Methyl Binding Proteins:-

Eukaryotic chromosomes are the repositories of the genetic information necessary to direct the synthesis of cellular components. Information is embedded in them at multiple levels. In addition to the genetic information contained in the sequence of nucleotide bases, chromosomes contain an `epigenetic code' that provides information crucial to regulation of the DNA itself. One component of this results from a system that covalently modifies cytosine residues by methylation at the 5 position of the pyrimidine ring (Bestor, 1990; Bird and Wolffe, 1999; Jaenisch and Bird, 2003). In almost all cases, mammalian DNA methylation occurs solely within the context of a simple palindromic sequence, CG, in which both cytosine residues are methylated. The methyl groups protrude into the major groove of DNA, providing novel functional moieties available for molecular interactions within this key surface of the double helix. The methylated fraction of the genome includes such interesting loci as imprinted genes, the inactive X chromosome, and transposable elements and their relics. These regions are strongly repressed and DNA methylation is believed to play an integral role in establishment and/or maintenance of this repression.

Methylation-mediated gene regulation is proposed to occur through the following two mechanisms. Firstly, CpG methylation within the transcription factor binding domain may directly interfere with the binding of certain transcription activators to the target sequence (Watt and Molloy, 1998, Takizawa et al, 2001). More generally, methylation-mediated gene silencing is through the action of a family of methyl-CpG binding proteins such as MeCP2 and MBD1, which preferentially bind to methylated CpG(s) (Lewis, et al., 1992, Cross, et al, 1997, Nan, et al., 1997).

At present, five proteins with mCpG-binding motifs have been identified in the MBD family, namely MBD1, MBD2, MBD3, MBD4 and MeCP2. All MBD proteins share a similar MBD of about 75 amino-acid residues. Sequence alignment of MBD proteins reveals several highly conserved residues on the interface between MBD and methylated DNA (mDNA). The conserved residues of the MBD form the interfacial surface contacting mDNA and are responsible for recognition of the methyl-CpG steps.

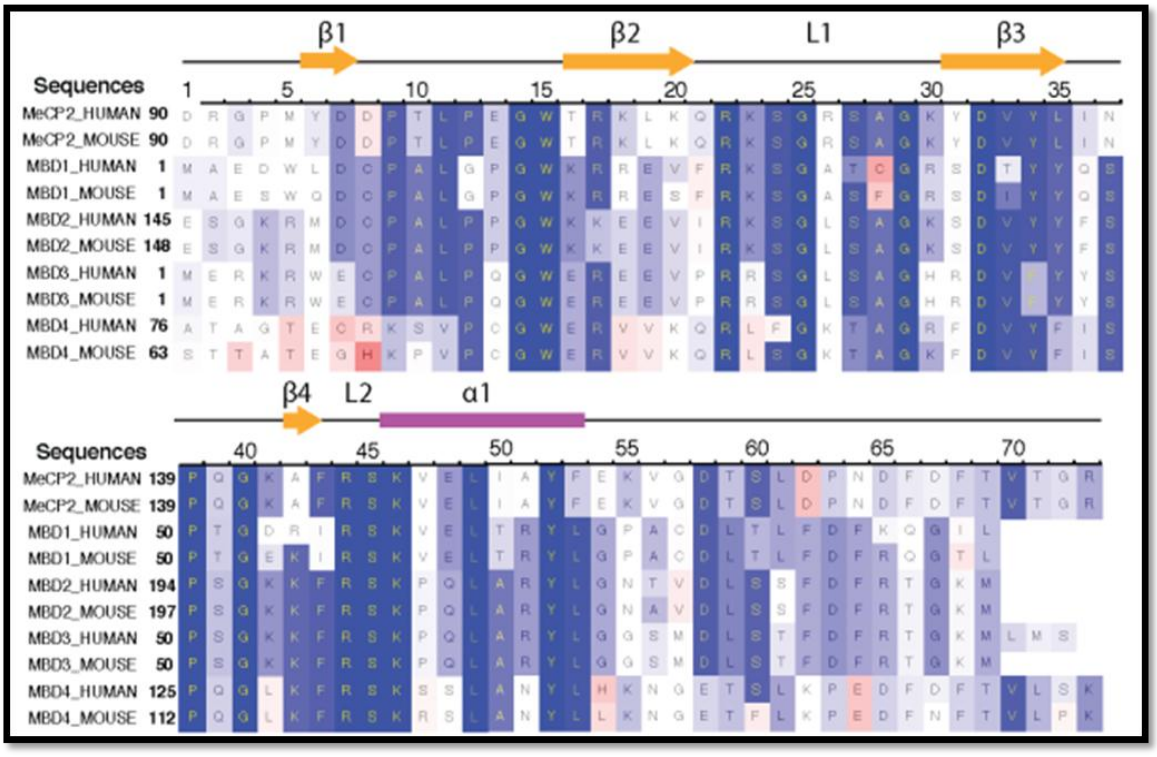


Fig 2: Sequence alignment of five methyl-CpG binding domain (MBD) proteins from human and mouse (Adapted from Bestor, T. H, Biological science, 1990)

Methyl-CpG–DNA binding proteins MeCP2, MBD1, MBD2, MBD3, and MBD4 comprise another distinct class of mammalian nuclear proteins (Ng, et al., 1999, Hendrich et al., 1999). Among these, MeCP2 is capable of binding to a single, symmetrically methylated CpG dinucleotide and binds to chromosomes at sites known to contain methylated DNA. MBD1 is a transcriptional regulator characterized by two or three CXXC domains that bind methylated CpG islands of the tumor suppressors p16, VHL, and E-cadherin genes and repress transcription (Patra et al,2001) MBD2 has been reported to possess DNA (cytosine-5) demethylase activity (Bhattacharya et al.,1999) along with repressor function [31,35,36]. MBD4 is a repair enzyme of the DNA-glycosylase family (Zhu et al., 2000) and MBD3 apparently lacks specific methylated DNA-binding capability although it has a high degree of sequence similarity with MBD2 (Hendrich et al,, Bhattacharya et al,, R.I.D et al., 1999). The finding that the hyper-methylation of promoters is associated with silencing of several genes in human prostate cancer is noted in the literature but the study of the respective enzymes and their relative levels, and repressor proteins is lacking.

Recently we have shown that DNMT1 and HDAC1 are up-regulated (Patra et al., 2001), and DNA demethylase activity and MBD2 protein expression are lacking (Patra et al.,2002)in human prostate cancer. We hypothesize that the packaging of methyl-CpG–DNA binding proteins (MBDs and MeCP2) and simultaneous translocation of histone Deacetylase (HDAC) to the hypermethylated segment of DNA is one of the mechanisms of remodeling/repression for hypermethylated genes by deacetylation of histones.

2.3. **The Methyl-binding Proteins: Linking DNA Methylation and Chromatin Structure:-**

MBD proteins bind to methylated CpG islands and mediate transcriptional repression of affected genes. The MBD family of proteins consists of five members: MeCP2, MBD1, MBD2, MBD3 and MBD4 (Berger, et al., 2005). The correlation between DNA methylation and transcriptional inactivity is well established. However, a causative role for CpG methylation in repression of transcription has often been a subject for debate. Although many silenced genes are associated with dense CpG methylation, this epigenetic mark could be a downstream consequence of transcriptional inactivity rather than an active participant in the process of

repression. However, the identification of a family of proteins that bind to DNA containing methylated CpG dinucleotides established a causative link between CpG methylation and repression of transcription. Five MBD family members have now been identified.

2.3.1. MBD1:-

MBD1 has an NH2-terminal MBD and a COOH-terminal TRD. In addition, full-length MBD1 contains three CXXC motifs similar to the motif present in Dnmt1. MBD1 binds preferentially to densely methylated DNA *in vitro*, and it represses transcription in a HDAC-dependent manner in transfected cells (Cross, et al., 1997, Ng, H.et al., 2000, Fujita, et al., 2000). Consistent with its *in vitro* DNA binding characteristics, overexpressed green fluorescent protein-tagged MBD1 localizes to densely methylated major satellite DNA in mouse cells (Hendrich, et al.,1998), and it is concentrated at methylated pericentromeric regions of chromosome 1 in human cells (Fujita,et al.,1999). Endog- Cell Growth & Differentiation endogenous MBD1 is detected along Euchromatic regions in human diploid metaphase chromosome spreads, but it concentrates at centromeric heterochromatic regions of chromosomes 1, 9, 15, and 16, as well as regions of densely methylated spacer DNA sequences interspersed among rRNA genes.

Furthermore, the intensity of MBD1 staining is generally inversely proportional to the staining intensity of acetylated histone H4 (66). In human cells, MBD1 mRNA is expressed as five alternatively spliced forms that encode isoforms differing in their COOH-terminal and CXXC regions. Although the functional consequences of these alternative forms are unclear, inclusion of all three CXXC motifs results in an MBD1 isoform capable of repressing transcription independently of DNA methylation in transfected cells.

2.3.2. MBD2:-

MBD2 exists as two differentially expressed isoforms (MBD2a/2b), of which MBD2a differs by a 152 AA N terminal extension (Hendrich and Bird, 1998). In several independent biochemical purifications of the MeCP1 complex, (Wade et al.,1999)MBD2 is proven to be a component of MeCP1 complex and is coupled with the Mi2/NuRD complex to mediate the gene

silencing effect of NuRD/MeCP1 complex directed to methylated genes (Zhang et al.,1999, Ng et al.,1999). MBD2 includes partially overlapping MBD and TRD domains (Boeke et al., 2000).

Consistent with an HDAC-dependent model of gene repression, the TRD exhibits TSA-sensitive transcription repression activity in reporter assays (Ng et al., 1999).The MBD binds to DNA with a single methylated CpG *in vitro* (Cross et al., 1997, Wade et al.,1999), and an MBD2-GFP fusion protein binds to major satellite DNA in transfected mouse cells (Cross et al.,1997). Furthermore, MBD2 has been reported to be associated with Sin3a (Boeke et al., 2000). It is probable that the variation in reported factors and complexes associated with MBD2 reflects several distinct yet overlapping cell context-reliant functions of this family of proteins.

2.3.3. MBD3:-

MBD3 has extensive sequence similarity to MBD2 (Hendrich and Bird., 1998). It is expressed as several splice variants, some of which disrupt the MBD (Zhang et al., 1999). MBD3 mRNA is expressed in the adult brain .In the developing brain, MBD3 expression can be detected in high levels in neuroepithelial cells whereasMBD2 is barely detected(Jung et al,2003).The protein has been identified as a component of the Mi-2/NuRD transcriptional co-repressor complex that includes Mi-2 ATPase, HDAC, and other proteins (Wade et al,1999, Humphrey et al,2001). However, *in vitro*, mammalian MBD3 has little if any methyl-CpG-binding activity, likely because of amino acid substitutions within the MBD (Hendrich et al, 1998). MBD3 can be transiently induced by kindling or transient ischemia in rodent hippocampus, suggesting that these proteins may be involved in alteration of gene expression upon lesions (Francis et al, 2002, Jung et al., 2002).Therefore, unlike the case for the *Xenopus* orthologue of MBD3 which contains a MBD with methyl-CpG-binding activity it is unlikely that mammalian MBD3 plays a role in methylation dependent transcription repression.

MBD3 is a necessary component for NuRD complex and involved in multiple gene regulatory pathways for embryogenesis (Hendrich et al., 2001). Future study using a strategy for brain-specific conditional knockout of MBD3 will shed light on the specific role of MBD3 in the nervous system.

2.3.4. MBD4:-

MBD4 includes a MBD similar to that of MeCP2, although the COOH-terminal domain is homologous to bacterial DNA repair enzymes (Hendrich et al, 1998). Although MBD4 is capable of binding to methyl-CpG sites, it has a higher affinity for 5mCpG-TpG mismatched sites (Hendrich et al., 1999), and the DNA repair domain provides DNA *N*-glycosylase activity at G-T mismatches (Hendrich et al., 1999, Petronzelli et al., 2000). Therefore, MBD4 is ideally suited to function in the repair of point mutations that result from spontaneous deamination of 5-methylcytosine to thymine. In addition, MBD4 (also known as MED1) binds to the MLH1 DNA mismatch repair protein *in vivo*. Expression of a MBD4 mutant lacking the MBD induces microsatellite instability in cell lines, implicating MBD4 in this form of DNA repair as well (Bellacosa et al., 1999). These data suggest that MBD4 may serve as a strand discrimination factor for MLH1, directing mismatch repair activity to the newly synthesized strand. However, in an *in vitro* assay, nuclear extracts containing MBD4 perform mismatch repair independently of target CpG methylation status (Drummond, et al., 2001).

MECP2 (methyl CpG binding protein 2 (Rett syndrome)) is a gene (Amir et al., 1999) that provides instructions for making its protein product, MECP2, also referred to as MeCP2 (Lewis et al., 1992) MECP2 appears to be essential for the normal function of nerve cells. The protein seems to be particularly important for mature nerve cells, where it is present in high levels. The MeCP2 protein is likely to be involved in turning off ("repressing" or "silencing") several other genes. This prevents the genes from making proteins when they are not needed. Recent work has shown that MeCP2 can also activate other genes (Chahrour M, et al. 2008).Within the brain, the MeCP2 protein is important for the function of nerve cells (neurons) and is present in high levels in mature neurons.

This protein likely plays a role in maintaining connections (synapses) between neurons, where cell-to-cell communication occurs. Many of the genes that are known to be regulated by the MeCP2 protein play a role in normal brain function, particularly the maintenance of synapses.

Table 1: Genomic localizations of methyl-binding proteins in different species of mammals

Species	MeCP2	MBD1	MBD2	MBD3	MBD4	Kaiso
Homo sapiens	Xq28	18q21	18q21	19p13.3	3q21–q22	Xq23
Mus musculus	XA7.3	18E2	18E2	10C1	6E3	XA3.3
Rattus norvegicus	Xq37	18q12.2	18q12.1	Not placed	4q42	Xq11
Bos taurus	X	24	Not placed	7	22	Not placed

2.4. MBD Proteins act on Gene Silencing:-

The MBD sequence motif was defined by molecular analysis of prototype of MBD protein and MeCP2 (Nan et al., 1993). This MBD sequence motif was found to be both necessary and sufficient to be direct specific interaction with methyl-CpG-containing DNA fragments. It consists of approximately 70 amino acids and represents the sole sequence feature found in common all of the MBD family members. The single exception to this rule is case of the MBD2 and MBD3 which are nearly identical in amino acid sequences, and have common gene structures and are believed to have arisen from an ancient duplication in all the evolution of the vertebrate lineage (Hendrich et al., 2003).

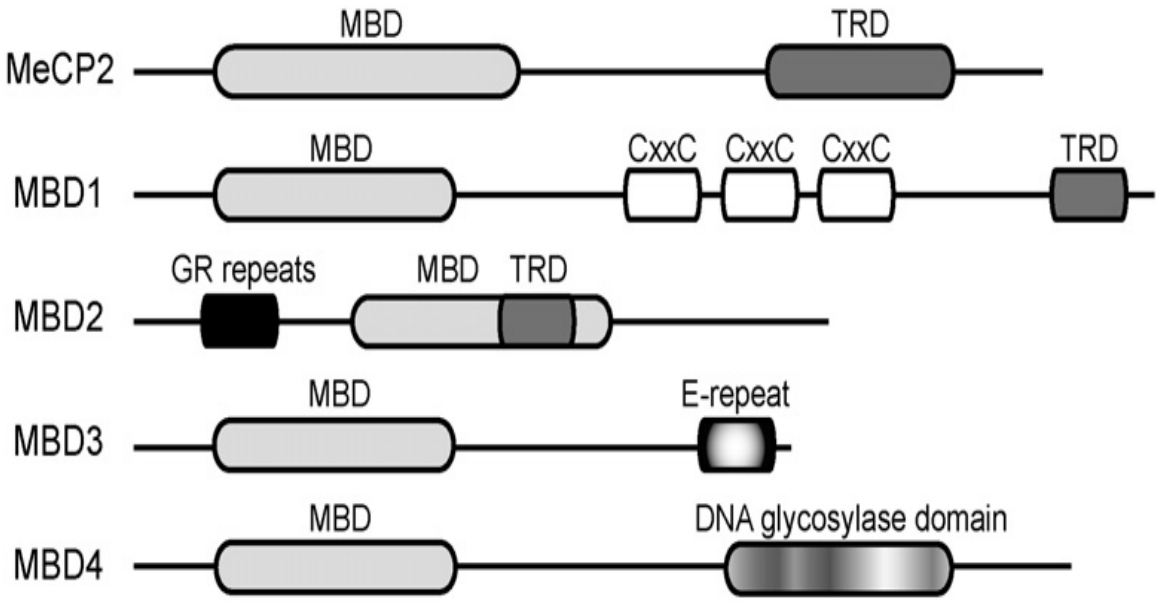


Fig.3: The figure shows the general structural domains of MeCP2 and the MBD family proteins. [MBD=methyl-CpG-binding domain; MeCP2=methyl-CpG-binding protein 2; TRD = transcriptional repression domain; CXXC = cysteine rich domain; GR=glycine and arginine repeats; E-repeat = glutamate repeat] (Dhasarathy and Wade, Journal of Biological essays, 2008).

The methylated fraction of this genome includes such interesting loci as imprinted genes and the transposable elements and inactive X chromosome, and their relics. These all regions are strongly repressed and DNA methylation is believed to be playing an integral role in establishment and/or maintenance of transcriptional repression. Interestingly, mammalian MBD3, unlike amphibian counterpart, fails to selectively recognize methylated DNA owing to substitution of a critical tyrosine residue within the MBD motif with phenylalanine (Fraga et al., 2003). Four members of the MBD family are to be believed to function, at least in part, in transcriptional repression (Bird and Wolffe, 1999; Hendrich and Tweedie, 2003; Wade, 2001).The fifth MBD protein, MBD4, has been DNA N-glycosylase enzymatic activity and probably functions in DNA repair (Hendrich et al., 1999). In most cases, all the MBD proteins are expressed ubiquitously (Hendrich and Bird, 1998; Meehan et al., 1992).

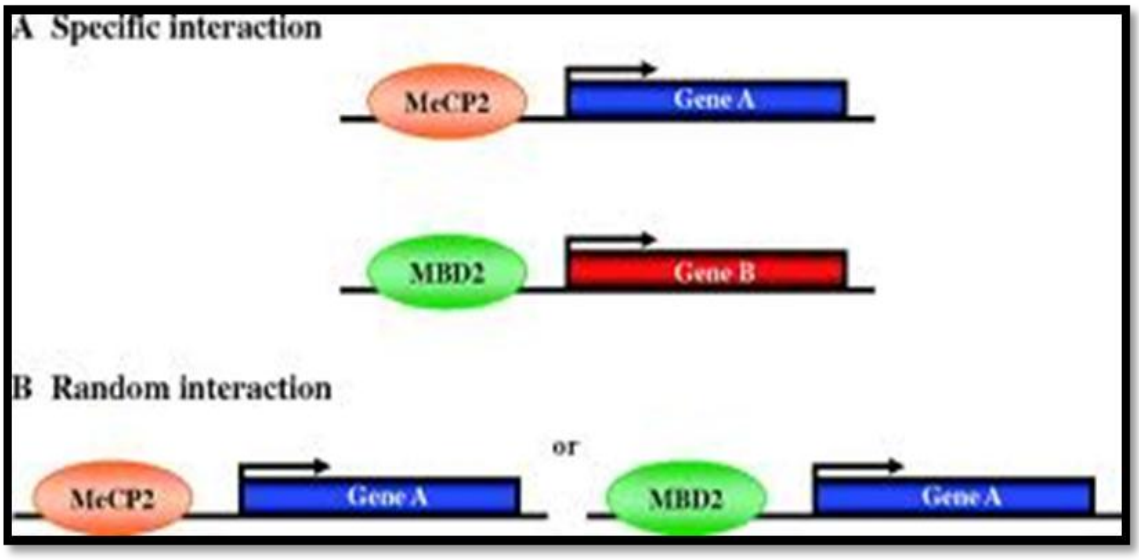


Fig 4: Two potential models for gene regulation by MBD family members (Mehrnaz Fatemi and Paul A. Wade, Journal of cell science, 2006)

A. In the specific interaction model, each methylated locus is associated with one and only one MBD family member.
B. In the random interaction model, the association of a given methylated locus with an MBD family member is random

2.4.1. Germline variants:-

Three polymorphisms in MBD1 and Pro401Ala) have been investigated for their association with lung cancer risk (Jang et al., 2005). Further subgroup analyses suggested that all three polymorphisms are associated with an increased risk of lung adenocarcinoma. The three polymorphisms are in strong linkage disequilibrium, and in vitro analysis of the promoter activity of the variants of 634G.A and 501delT attributed much of the functional effect to lower MBD1 expression from the G allele of 634G.A.For MBD2 Zhu et al. (37) found a reduced risk of premenopausal breast cancer for the rare variants of two SNPs (rs1259938 and rs609791). No association was observed in postmenopausal women. (Cebrian et al., 2006) found no evidence of an association between SNPs or haplotypes in MBD2 and breast cancer risk in a similar population in a study that had more than 10 times the sample size of Zhu et al., but sub-group analyses by menopausal status were not reported. In MBD4, the Lys/Lys genotype was reported to increase the risk of esophageal squamous cell carcinoma by Hao et al., 2004).

2.4.2. Somatic changes:-

The role of somatic alterations in MBD proteins in cancer is unclear. It has been shown that MBD2 binds to the aberrantly methylated promoter of tumor suppressor genes (e.g. p14/ ARF and p16/Ink4A in colon cancer cell lines) and suppresses their expression (Magdinie et al., 2001). Abnormalities in the expression level of MBD proteins in cancer cell lines, however, may be due to the increased cell proliferation in these cells (Sato et al., 2002). MECP2 over expression has also been observed in breast cancer and appears to be associated with estrogen receptor positivity (Muller et al., 2003). The study of Mbd2-deficient mice crossed on to an Apc Min/þ background has shown that these mice are resistant to the development of intestinal tumors and the dosage of Mbd2 is important for the tumor resistant effect (Sansom et al.,2003).

2.5. Mechanisms of MeCP2-mediated gene silencing:-

MeCP2 protein binds methylated CpG sites at gene promoters and promotes gene silencing by associating with Sin3A and histone deacetylases to form co-repressor complexes and through its interaction with CREB selectively regulates active BDNF gene transcription (Akbarian & Huang, 2009; Chahrour et al., 2008; Chen et al., 2003; Klose & Bird, 2003; Nan et al., 1998). Rett syndrome is an X-linked neuron developmental disorder, which primarily affects females and leads to postnatal lethality

in males (Amir et al., 1999). Patients with Rett syndrome suffer from severe motor and cognitive impairments and may often display autistic features. Rett syndrome is due to a mutation in the MeCP2 gene. Thus, MeCP2 mutation leads to abnormal regulation of genes necessary for proper development and cognitive function. Interestingly, recent work suggest that MeCP2 functions not only to repress gene transcription but to mediate activation of genes as well (Chahrour et al., 2008 and see Fig. 3, Gupta et al., 2010) all in the context of memory formation. These studies provide us with new insights into the gene targets that could be deregulated due to loss of MeCP2 function in Rett syndrome.

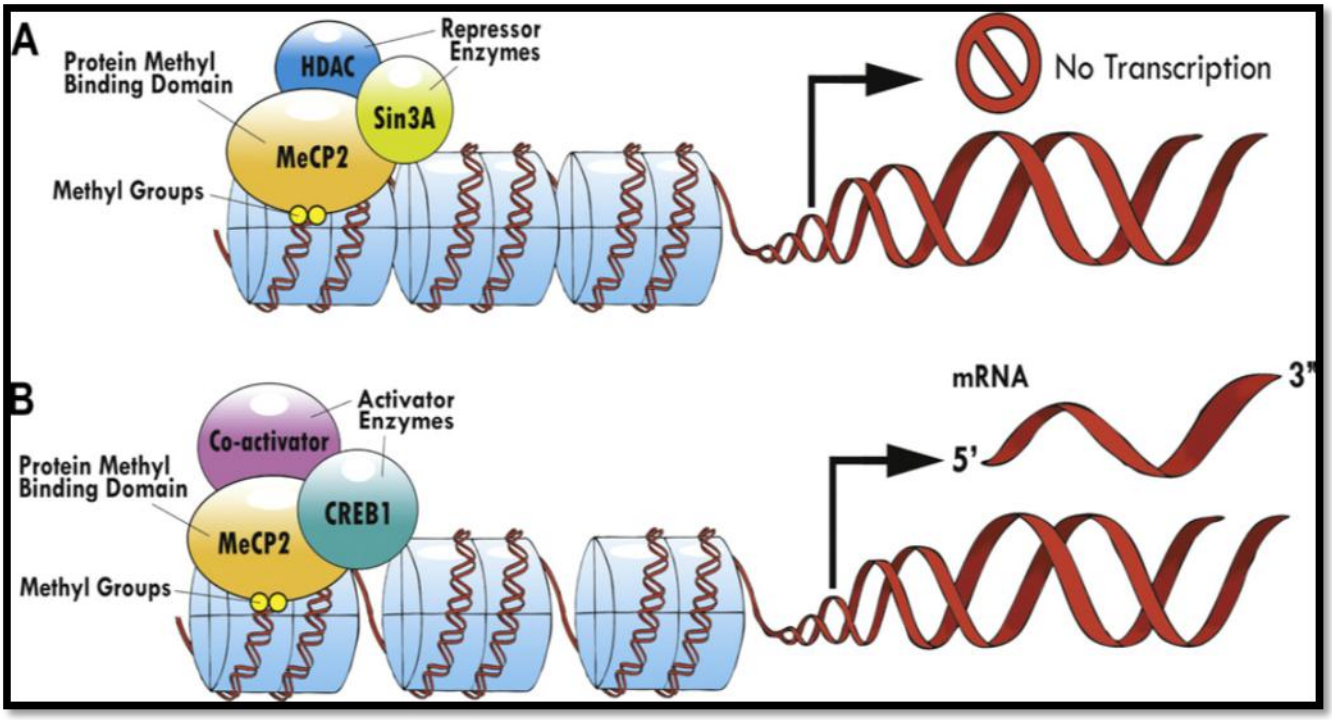


Fig 5: Mechanisms of MeCP2-mediated gene silencing and transcriptional activation. (Farah D. Lubin, 2011)

A. Generally, methyl-DNA binding domain (MBD) proteins such as MeCP2 are recruited to methylated CpG sites and assemble repressor enzymes to mediate repression of gene transcripts.
B. Although this is a well-characterized role of MBD proteins in transcriptional regulation, it has been reported that the MeCP2 MBD protein can also be associated with transcriptional activation via CREB1.

2.6. Action of MBD proteins in cancer:-

It has recently been recognized that cancer is a manifestation of both abnormal genetic and epigenetic events (Jones, 2003). Deregulated epigenetic controls, which usually are represented by abnormal DNA methylation patterns such as global hypomethylation and region specific hypermethylation, are a hallmark of most cancers. Aberrant hypermethylation of promoter CpG islands and the resulting transcriptional silencing is nowadays a widely accepted mechanism of inactivation of tumor suppressor genes in cancer that actively contributes to tumorigenesis (Jones and Laird, 1999; Costello and Plass, 2001; Esteller, 2002; Herman and Baylin, 2003). Treatment of cancer cells with the demethylating agent 5-aza-2′-deoxycytidine

results in CpG island hypomethylation, MBD release, and gene re-expression, reinforcing the notion that association of MBDs with methylated promoters is methylation-dependent. Whereas several promoters are highly specific in recruiting a particular set of MBDs, other promoters seem to be less exclusive. The existence of these profiles provides a powerful set of markers for outlining the disruption of critical pathways in tumorigenesis and for deriving sensitive molecular detection strategies for virtually every human tumor type.

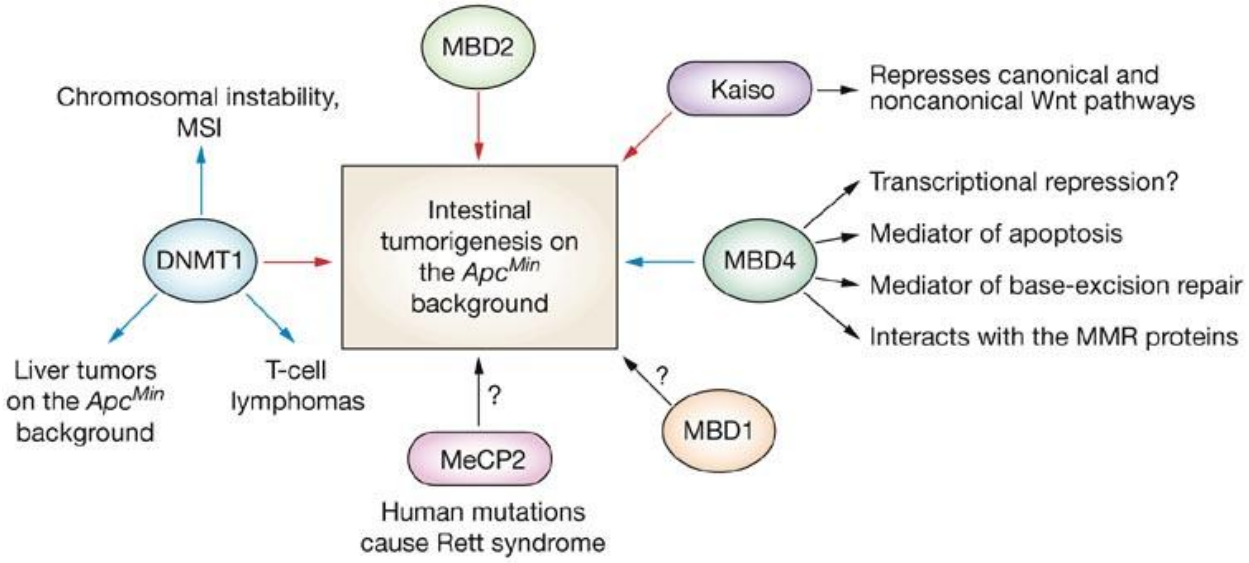


Fig 6: Action of MBD Proteins and Tumorigenesis
(Owen et al., Nature Clinical Practice Oncology (2007))

The systematic study of DNA methylation patterns in human cancer cell lines (Smiraglia et al.,2001,Paz et al.,2003*)* has shown that these are appropriate models for this type of study as they show methylation patterns that resemble their corresponding tumor types . The selectivity of MBDs for methylated DNA and their transcriptional repression properties suggest that they could exert their function in methylated promoters. The presence of MBD proteins in the methylated promoter of a gene in cancer was first shown in 2000, when Magdinier and Wolffe *(*Magdinier and Wolffe, 2001*)* identified MBD2 in the methylated promoter of $p16^{INK4a}$ in colon cancer cells. Human MBD genes are considered housekeeping genes because they are widely expressed in somatic tissues. Given the epigenetic role of MBD proteins in regulating gene expression, MBDs may be involved in cancer development by affecting the expression of cancer related genes. In fact, there is growing evidence that aberrant expression of MBD proteins is

associated with human cancers (Patra et al., 2003, Schlegel et al., 2002). The *MBD2* gene is mapped to the conserved region within human chromosome 18q21 (Hendrich et al., 1999).

A recent finding also suggests that MBD2 has potential DNA demethylase activity (Bhattacharya et al., 1999), implying that it might mediate gene activation in addition to transcriptional repression. However, two subsequent studies could not demonstrate any demethylase activity of MBD2 (Ng et al., 1999, Wade et al., 1999), and this inconsistency in the functions of MBD2 remains to be resolved. So several studies in human cancer research have demonstrated that the MBD2 protein plays a role in tumorigenesis. Moreover, a significant reduction in MBD2 mRNA expression was found in human colorectal and gastric cancerous tissues (Kanai et al., 1999) and peripheral blood lymphocytes (Zhu et al., 2004) in bladder cancer patients, implying a protective role for MBD2 in tumorigenesis. MBD2 protein expression and its demethylase activity were detected in normal human prostate tissue but not in cancerous tissue (Patra et al., 2002). These differences between types of cancers in the abundance of MBD2 levels may reflect different roles for MBD2 either in transcriptional repression or in the demethylation process.

2.7. Epigenetic Signature Using MBD Proteins:-

In cells and tissues, MeCP2 has been associated with gene silencing that appears to be local and mediated through recruitment of histone modification enzymes (W.G. Chen., et al, 2003, K. Martinowich, et al., 2003). However, some data indicate that the protein itself is not strongly associated with such activities in the mammalian brain (K. Hu, et al., 2006). There are reports of MeCP2-dependent higher-order chromatin structures associated with imprinted gene clusters (Horike et al., 2005) although these have also been questioned (Schule et al.,2007). In addition, MeCP2 has been implicated as an RNA binding protein involved in splicing of mRNA (Young et al., 2005). Finally, the question of where the protein is bound *in vivo* and what genes it associates with casts doubts on long-held models (Yasui, et al., 2007). The MBD protein family has been proposed as potential readers of the DNA methylation mark. Yet it is clear that the phenotypic consequences of mutation of the system for deposition of DNA methylation are far more profound than are the consequences of mutation of the MBD proteins themselves (Bird, 2006). The different members of the DNA methyltransferase family are essential in mammals; null mutants in MBD family members in mice are largely viable and fertile.

RATIONALE BEHIND THE PROJECT

3. RATIONALE BEHIND THE PROJECT

Proteins with a methyl-CpG-binding domain (MBD) can bind to single methylated CpGs and most of them are involved in transcription control. So far, five vertebrate MBD proteins have been described as MBD family members: MBD1, MBD2, MBD3, MBD4 and MECP2.

Keeping this hypothesis in mind and based on the literature survey, the present work has been undertaken to investigate whether four methyl binding candidate proteins have putative transcriptional repression activity in vitro in cancerous tissue.

The objectives of this project include:-

- Comparative analysis of RNA isolation from three different RNA Purification techniques.

- Comparative qualitative analysis of the expression level of Methyl Binding proteins – MBD1, MBD2, MBD3 and MBD4 in normal and leukemia Sample.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1 Collection of Samples:

Blood was collected as the normal tissue from the local CWS Hospital, Rourkela, Odisha, stored in ice and immediately processed for better RNA extraction. Cancer tissue (Lymph Node Carcinoma) was collected from National Medical College, Kolkata and stored in RNA later (Sigma) at -20^0C until the extraction of RNA.

4.2 Extraction of Total RNA:

Total RNA was extracted from blood (normal) and cancer tissue using GeneJET^{TM} RNA Purification Kit (Fermentas), Manual RNA Extraction techniques and Trizol method.

4.2.1 Extraction from Blood By RNA Purification Kit:-

The collected blood was centrifuged at 3000 rpm for 15 mins at 4° C. The supernatant containing the serum was separated from the pellet which contains the blood cells. The pellet was resuspended in 600 µl of Lysis Buffer (supplemented with 20 µl of 14.3 M β-mercaptoethanol for every 1ml of Lysis Buffer) and vortexed to mix thoroughly. 450 µl of ethanol (96-100%) was mixed with the solution. About 700 µl of the lysate was transferred to a GeneJET^{TM} RNA Purification Column inserted in a collection tube and centrifuged at 12000 rpm for 1 min at 4° C. The flow-through was discarded and the column was placed into a new 2 ml RNase-free micro centrifuge tube. 700 µl of Wash Buffer 1(supplemented with 250 µl of ethanol for every 1ml Wash buffer 1) was added to the column and centrifuged for 1 min at 12000 rpm. The flow-through was discarded and 600 µl of Wash Buffer 2 (supplemented with 850 µl of ethanol for every 0.5 µl Wash buffer 2) was added to the column. It was centrifuged at 12000 rpm for 1 min at 4° C. The flow-through was again discarded. Centrifugation was again done at 12000 rpm for 1 min at 4 ° C by adding 250 µl of Wash buffer 2. The flow-through was discarded and the column was transferred to a sterile 1.5 RNase-free micro centrifuge tube. 100

µl of nuclease-free water was added to the column and centrifuged for 1 min at 12000 rpm to elute RNA. The RNA was stored at - 20° C for further use or immediately processed for cDNA synthesis.

4.2.2. mRNA Extraction From Blood by Trizol RNA Extraction techniques:-

The collected blood was transferred in a 2 ml tube with 1 ml Trizol. Then it was homogenized for 60 seconds in the polytron. 200µl of chloroform was added. Mixed by inverting the tube for 15 seconds. Incubated for 3 min at room temperature. Centrifuged at 12.000 g for 15 minutes, Transferred the aqueous phase into a fresh Eppendorf tube.500µl of isopropanol was added. Centrifuged at maximum 12000 g for 10 minutes. Then the pellet was washed with 500µl 70 % ethanol .Centrifuged at max. 7500 g for 5 min in the cold room .Dried the pellet on air for 10 min .Dissolve the pellet in 50-100µl DEPC-H2O Incubated for 10 min at 60 ^{0} C .Took spectrophotometer reading .And analyzed the RNA on a MOPS gel. Then 1-3µg RNA was dissolved in 11µl denaturation buffer. 1µl ethidium bromide (1mg/ml) was added and denaturated at 65 ^{0}C for 15 minutes. Then 1 % agarose gel was added in MOPS buffer plus 5 % formaldehyde. Then the gel was run at 40 V for 4 hour.

4.3 Quantitative Estimation of RNA Concentration by Spectrophotometric Analysis:

The concentration of the extracted total RNA from both blood and cancer tissue was quantified by measuring the absorbance at 260 nm in a spectrophotometer (ELICO, BL 200 Bio Spectrophotometer, double beam) and calculated by using the formula as given below:

$$\text{Total RNA } (\mu g/ml) = OD_{260} \times 40 \times \text{Dilution factor.}$$

4.4 Quantitative Estimation of RNA Concentration by Denaturing Gel Electrophoresis:

The extracted RNA from both blood and cancer tissue was run on a denaturing agarose gel and the quantity of RNA estimated from the band intensity. For denaturation gel (40 ml), 0.6 g agarose (Sigma), 28.8 ml dH_2O (Sigma), 7.2 ml formaldehyde (Sigma), 4 ml 10X MOPS

buffer were mixed properly. About 2 µl (2µg) of the total RNA was mixed with 18 µl 1X Reaction Buffer (2µl of 10X MOPS Buffer, 4 µl formaldehyde, 10 µl formamide (Sigma) ,2 µl 0.2 mg/ml Etbr (Sigma)) and incubated at 55 °C for 1 hr. It was then cooled on ice and loaded in the wells of the denaturing gel.

4.5. First strand cDNA synthesis:

Total RNA (4 µg) from both blood and cancer tissue were used for first strand cDNA synthesis by reverse transcription using RevertAid^{TM} First Strand cDNA Synthesis Kit (Fermentas) in a thermocycler (Biorad). The RNA were incubated with 1 µl of oligo (dT) primers (100 µM, 0.2 µg/µl) and 12 µl of nuclease-free water at 65 °C for 5 min. The reaction was cooled on ice to allow the primers to anneal to the RNA, then spin down and placed on ice again after which the following components were added to the reaction in order; 4 µl of 5X Reaction Buffer, 1 µl of Ribolock^{TM} RNase inhibitor (20 U/µl), 2 µl of 10 mM dNTPs and 1.0 µL of RevertAid^{TM} M-MuLV-Reverse Transcriptase (200 U/µl). The reagents were gently mixed and incubated for 1 hr at 42°C. Heating at 70°C for 5 min terminated the reaction and the synthesized cDNA was stored at –20 ^{0} C for further use.

4.6. Gene-specific PCR for amplification of the desired gene:-

4.6.1 Selection of Primers:

A set of specific forward and reverse primers for the amplification of the desired gene under study was selected from published papers (Patra et al., 2003; Zou et al., 2002). The cDNA of both the blood and cancer tissue synthesized were used as the template for the specific primers. The constitutively expressed housekeeping gene, β-actin was used as a positive control to ensure high quality. The primer sequences used for the PCR reaction are shown in Table 4:

Table 2: Table showing the sequence of the forward and backward primers.

PRIMER	TYPE	SEQUENCE
MBD1	***Forward***	5' CACCCTCTTCGACTTCAAACAAG 3'
	Reverse	5' CAACCTGACGTTTCCGAGTCTT 3'
MBD2	***Forward***	5' AACCCTGCTGTTTGGCTTAAC 3'
	Reverse	5' CGTACTTGCTGTACTCGCTCTTC 3'
MBD3	***Forward***	5' CCGCTCTCCTTCAGTAAATGTAAC 3'
	Reverse	5' GGCTGGAGTTTGGTTTTCAGAA 3'
MBD4	***Forward***	5' TGGTGGTGCATGCCTGTAAT 3'
	Reverse	5' TGAGACAGGGTCTCTCTCTGTCAT 3'
β-ACTIN	***Forward***	5' TCTACAATGAGCTGCGTGTG 3'
	Reverse	5' TCTCCTTCTGCATCCTGTC 3'

4.6.2 PCR conditions:

The PCR sample mixtures, in a 25 µl volume, contained 17 µl of dH_2O (Sigma), 2.5 µl of 1X PCR buffer (Sigma), 0.5 µl of dNTP (0.2 mM, Sigma), 1.5 µl of $MgCl_2$ (1.5 mM, Sigma), 0.5 µl each of the forward and reverse primers (0.2 µM, Sigma) of MBD1, MBD2, MBD3, MBD4 and 0.5 µl Taq DNA-polymerase (1U/µl, Himedia). 2 µl of each cDNA sample was added. PCR amplifications of MBD1, MBD2, MBD3 and MBD4 were performed in a thermal cycler by initial denaturation at 94° C for 1 min, followed by 30 cycles of denaturation at 94° C for 20 secs, annealing at 57 ° C for 20 secs, and extension at 72° C for 30 secs, followed by an final extension step at 72° C for 5 mins. For amplification of cDNA , the following conditions were followed: initial denaturation at 95° C for 5 mins, prior to 30 cycles of denaturation at 94° C for 30 secs, annealing at 57 ° C for 20 secs, and extension at 72° C for 45 secs, followed by an final extension step at 72° C for 10 mins.

4.7 Agarose Gel Electrophoresis of the PCR products:-

The generated PCR products were analyzed by electrophoresis on 1.5% agarose gel. Agarose gel was prepared with 1X TAE (Tris Acetate EDTA, Sigma) buffer. Before casting 0.1% ethidium bromide was added to the gel. 15 µl of sample (PCR product) was loaded to each well along with 3 µl 1 X loading dye. 5 µl of DNA marker (1 kb, Sigma). The gel was run in TAE buffer at 100 volt for 40 minutes.

4.8 Analysis of the Relative Expression level of the different genes:

The relative levels of expression of each gene were analyzed by taking the absorbance through spectrophotometric readings. The ratios of desired genes/β-actin product were subsequently calculated after subtraction of the background pixel intensity for each gene of interest and used to assess the differences in expression levels between normal and leukemia sample.

RESULT AND DISCUSSIONS

5. RESULTS AND DISCUSSION

5.1 Quality Check of RNA Isolated from Normal And Cancer Samples from three different RNA isolation methods:-

The concentration of the extracted total RNA from normal tissue and leukemia sample was estimated by taking OD_{260nm}/OD_{280nm}, which was found to be 572 µg/ml for normal tissue and 409 µg/ml for leukemia sample and then run on gel. Further integrity of the RNA samples was checked by RT-PCR using β-Actin primer pairs. Strong amplification products of β-Actin was found for all the genes in both normal and cancer tissue.

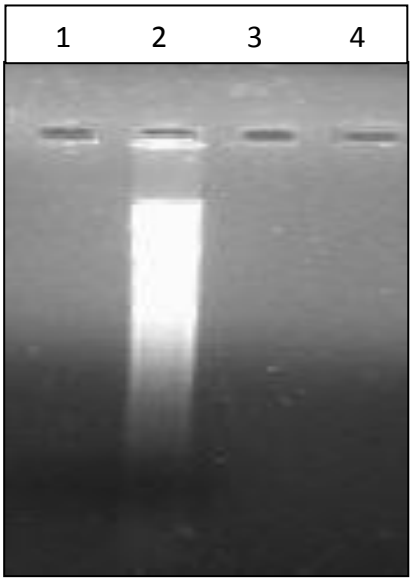


Fig 7: The photograph of total RNA extracted from leukemia sample run in 1% agarose gel.

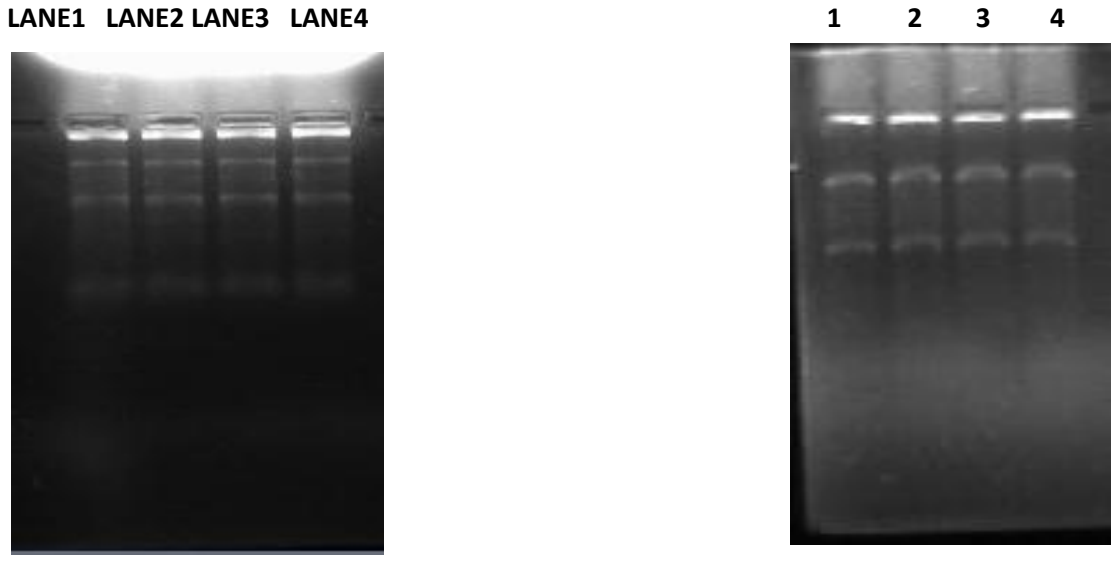
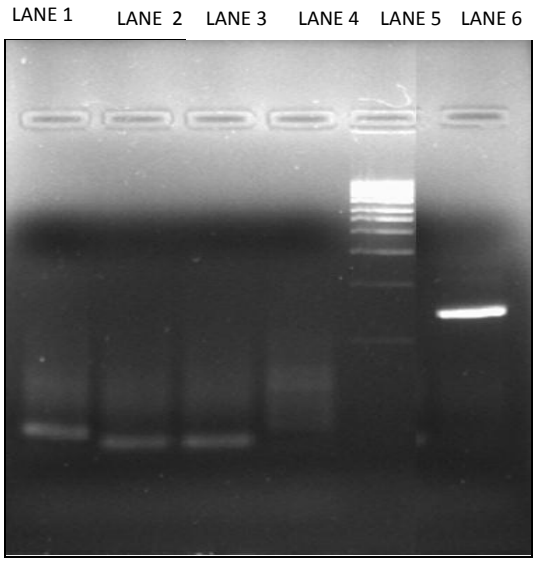


Fig 8: The photograph of total RNA extracted from (A) leukemia blood and (B) normal blood run in 1.5% denaturing gel.

5.2 Gene Specific Amplification:-

The PCR products obtained by gene specific amplification were run in a 1.5% agarose gel (Fig 9) and the concentration was estimated by spectrophotometric reading. The results obtained are given below. The expression of β-actin (682 bp) proves the integrity of the RNA extracted.

The gene expression of MBD1, MBD2, MBD3, MBD4, in leukemia blood were analyzed. MBD1 is a transcriptional repressor characterized by two or three CXXC domains that bind methylated CpG islands of the tumor suppressors' p16, VHL, and E-cadherin genes. MBD2 has been reported to possess DNA (cytosine-5) demethylase activity along with repressor function. MBD4 is a repair enzyme of the DNA-glycosylase family and MBD3 apparently lacks specific methylated DNA-binding capability although it has a high degree of sequence similarity with MBD2. In the present study, the expression of MBD1, MBD2, MBD3 and GADD45 were seen, hence it can be said that while MBD1 (125 bp), MBD2 (101 bp), MBD3 (101 bp) were expressed in cancer tissue, MBD4 was not expressed. Expression of MBD2 might point towards a suspected demethylase activity by MBD2.



LANE 1 - MBD1

LANE 2 - MBD2

LANE 3 - MBD3

LANE 4 - MBD4

LANE 5 - MARKER (1.5 kb)

LANE 6 - β-ACTIN

Fig 9: The photograph of the different genes from leukemia sample after gene-specific amplification seen in a 1.5% agarose gel.

On investigating the expression of other epigenetically modulating genes (fig 10) like HDAC, p300, it was compared with MBD group of proteins.

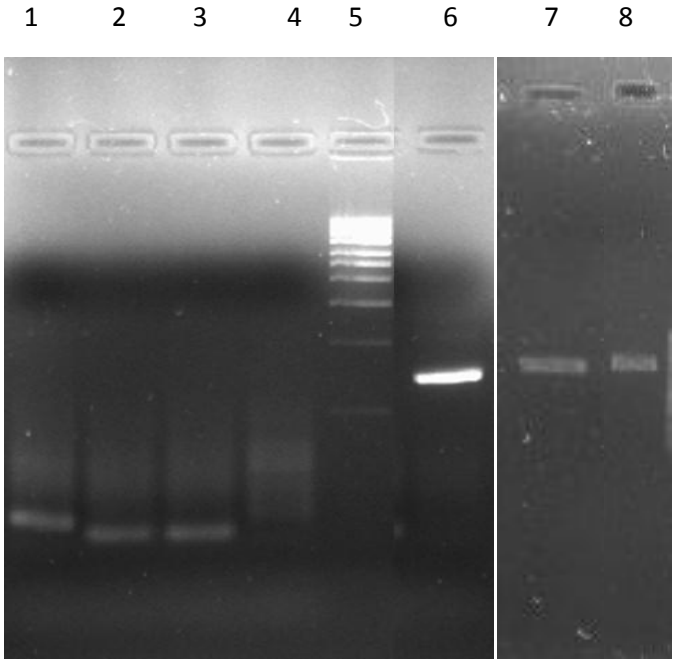


Fig 10: The gel photograph showing the different genes from cancer tissue in different lanes, 1-MBD1, 2-MBD2, 3-MBD3, 4-MBD4, 5-MARKER, 6-β-ACTIN,7-p300,8-HDAC.

5.3 Expression analysis of the desired genes:-

After the gene-specific amplification of the given genes was done for both normal blood and leukemia sample, the concentration was checked by taking its $OD_{260/280}$ in spectrophotometer. The following results were obtained (Table 2) and the data was plotted in the form of graph to do a comparative analysis of the expression level of the different genes.

TABLE 3: The table shows the concentrations of PCR products after gene-specific amplification.

Gene	***Concentration (µg/ml) at 260/280 nm normal blood***	***Concentration (µg/ml) at 260/280 nm leukemia blood***
MBD1	1.436	1.786
MBD2	1.064	1.238
MBD3	1.098	1.435
MBD4	1.176	1.584
HDAC	1.512	1.746
P300	1.543	1.864
β-actin	1.525	1.882

The graphs were plotted (Fig 11,12) according to the data given above and the following results were obtained.MBD4 shows highest gene expression in comparison to MBD1,MBD2,MBD3 and the β-actin gene which is a constitutively expressed house-keeping gene which indicates that the leukemia sample shows increased demethylation activity.

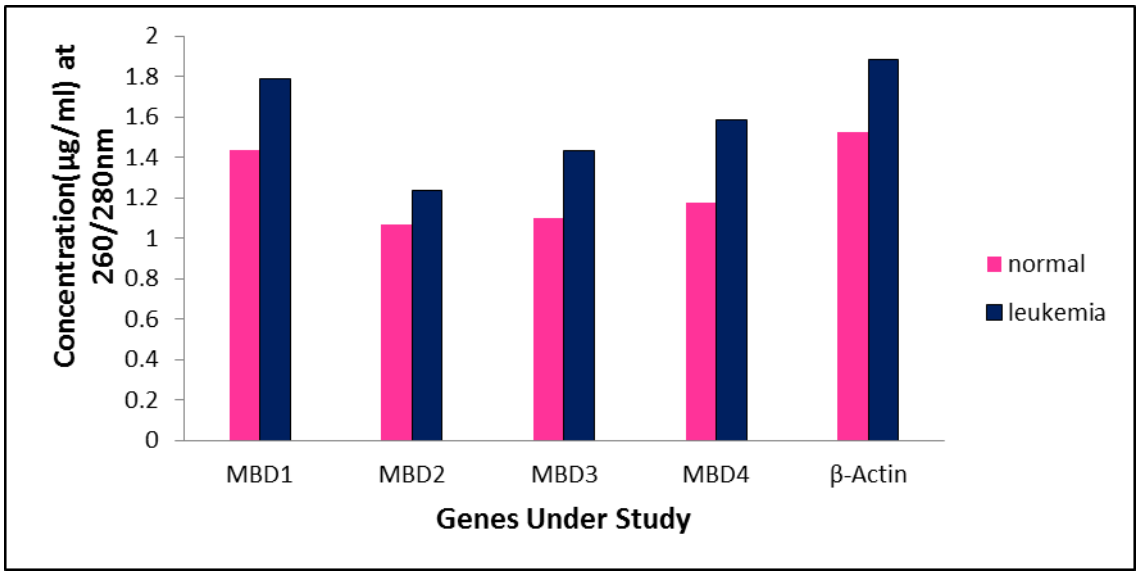


Figure 11. Graph showing comparative study of expression level of the genes under study (MBD1, MBD2, MBD3, MBD4 and as well as β-actin in both normal & leukemia sample.

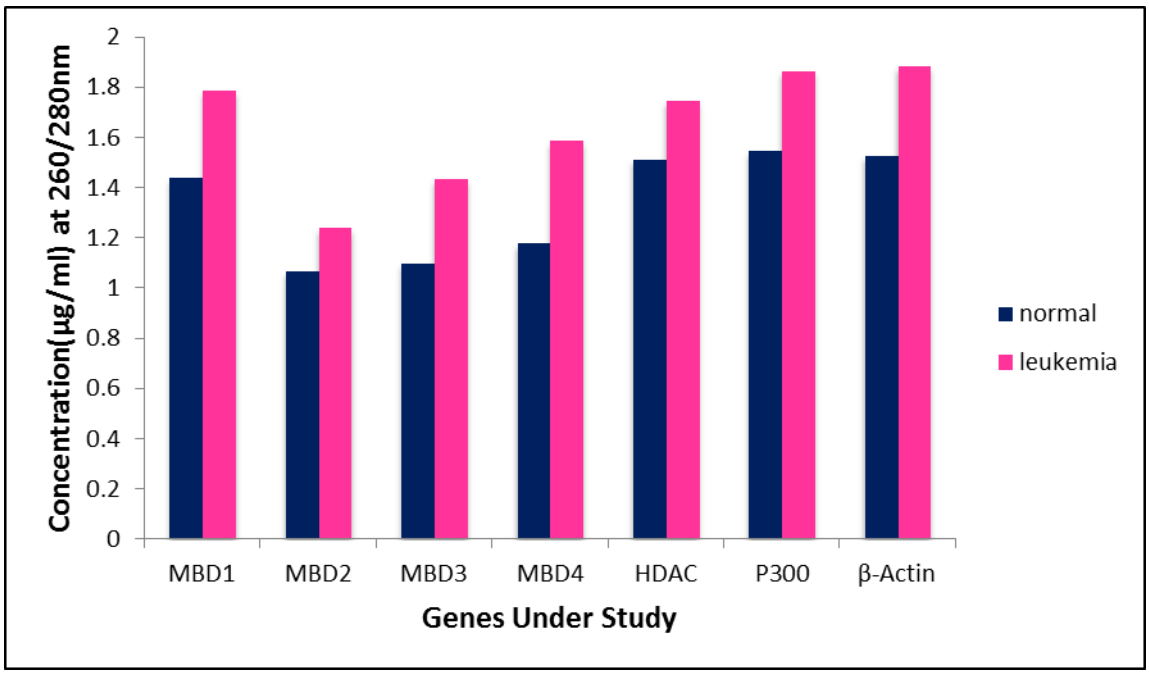


Figure 12. Graph showing comparative study of expression level of MBD proteins and HDAC, p300, as well as β-actin in both normal & leukemia sample.

CONCLUSION

CONCLUSION

In the present study, a comparative analysis of the expression of the different MBD proteins was studied in normal as well as cancer (leukemia) sample. We have observed, the expression of MBD1, MBD2, MBD3, hence it can be said that MBD1 (125 bp), MBD2 (101 bp), MBD3 (101 bp) might have some function in leukemia, whereas, MBD4 don't have expression. Thus it can be concluded that MBD proteins expression have a cause in the leukemia. This indicates that they may be associated with DNA hypermethylation mediated gene silencing in cancer. Correlating the expression of MBD proteins with other epigenetic regulators such as HDAC, p300, it was seen that the expression of MBD1, MBD2 and MBD3 is comparable to HDAC. Thus, in the epigenetic context, DNA methylation and Histone deacetylation work in tandem to transcriptionally silence the genes in asscociation with the MBD group proteins. The expression of MBD2 is very low which substantiates the previous studies that report a suspected demethylase activity of MBD2.

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